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MULTIPLE GENE BLOTTING TO SCREEN FOR CYTOKINE RNA EXPRESSION APPLICATION TO ENDOTHELIUM CELLS

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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EXPERIMENTAL DESIGN: Amplification products from polymerase chain reactions (PCR) of different cytokine genes were spotted onto nylon membranes, and probed with radioactive cDNAs made from mouse endothelioma cells.
RESULTS: The presence of interleukin-3 was detected, and confirmed by conventional Northern blotting and polymerase chain reaction (PCR).
CONCLUSIONS: This method may be useful to survey cells for the expression of cytokine genes.

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Multiple Gene Blotting to Screen for Cytokine RNA Expression: Application to Endothelioma Cells

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Abstract

BACKGROUND: Because normal and tumor cells can secrete different cytokines, it has been a matter of educated guesswork to select which factors to study. Nevertheless, many can be missed, so we have developed a method to screen for unanticipated cytokine expression.

EXPERIMENTAL DESIGN: Amplification products from polymerase chain reactions (PCR) of different cytokine genes were spotted onto nylon membranes, and probed with radioactive cDNAs made from mouse endothelioma cells. RESULTS: The presence of interleukin-3 was detected, and confirmed by conventional Northern blotting and polymerase chain reaction (PCR).

CONCLUSIONS: This method may be useful to survey cells for the expression of cytokine genes.

Keywords: Assay/blot/cytokine/expression/screening

Introduction

A formidable problem in the study of cytokines is the determination of factors produced by single or different types of cells. Many cytokines have been described, each interacting with others. Since there are multiple species, where does one start to look? Currently, many investigators make an educated guess, and test for certain cytokines individually. This is not only a laborious process, but has the disadvantage of potentially missing important cytokines.

To address this problem, we have developed an approach to screen for multiple cytokines using a single mRNA extract from cells or tissues. In this method, polymerase chain reaction (PCR) products from multiple cytokine genes are spotted onto nylon membranes, and probed with labeled cDNA made from total mRNA extracted from the cells of interest. As a model, we screened a mouse endothelioma (EOMA) cell line (1) for unknown cytokine gene expression, and were surprised to detect the presence of large quantities of interleukin-3 (IL-3) mRNA. This result was confirmed by standard Northern blot and PCR.

Experimental Design and Methods

Conditioned medium was prepared from $5x10^6$/ml rat spleen cells incubated with 5μg/ml concanavalin A (Sigma Chemical, St. Louis, MO) in RPMI-1640 medium plus 20mM HEPES, 1mM sodium pyruvate, 100U penicillin and 100ug streptomycin. After 48 hours at 37°C, the supernatant (CAS) was collected (5). This fluid typically contains about 250U/ml interleukin-2, 12U/ml interleukin-6, 6,000U/ml tumor necrosis factor, and some interleukin-5 and interferon-γ, but no detectable interleukin-1 or macrophage colony stimulating factor (6).

A final volume of 10% of this conditioned medium was added to a mouse endothelioma (EOMA) cell line (strain 129, H-2b) obtained from Robert Auerbach (1), to induce cytokine

production. After 2 hours, their RNA was prepared with RNA STAT-60 (TEL-TEST "B", Inc. Friendswood, TX) (7). Mixed cDNA probes were made with a SuperScript kit (GIBCO/BRL, Gaithersburg, MD) with 5-10μg RNA, 25ng random hexamers, and α-^{32}P-dATP (3000 Ci/mmol, Dupont NEN, Boston, MA) substituted for non-radioactive dATP (8).

To make the multiple cytokine blots, polymerase chain reaction (PCR) was performed on multiple human cytokine genes with Amplimers™ and conditions from Clontech (Palo Alto, CA). The PCR products were equalized visually by band intensity on agarose gel electrophoresis, and about 100ng was applied to each spot on a positively-charged nylon membrane (Nytran, Schleicher and Schuell, Keene, NH). The DNA was fixed to the membranes by ultraviolet light (Stratalinker, Stratagene, La Jolla, CA). The radioactive probes were hybridized to these blots at 42°C overnight and washed at 57°C by standard methods (9). The blots were exposed to Kodak XAR film with an intensifying screen at -70°C.

To confirm detection, EOMA RNA (15μg/lane) was electrophoresed in a 1% agarose-formaldehyde gel (9), and transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL). The blot was baked, and probed with a radioactive PCR product for mouse interleukin-3 and a mouse housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) (9) at 42°C. The blots were washed and autoradiographed as above.

For cytokine confirmation by PCR, 4μg EOMA RNA was primed with 25ng oligo-dT in the SuperScript kit to make cDNA. Then 5' and 3' Clontech amplimers for mouse IL-3 and G-3-PDH, at final concentrations of 0.4 μM each, were used for 100μl PCRs on a thermal cycler (MJ Research, Watertown, MA). The mixtures were heated at 95°C for 2 min., followed by 95°C for 40 sec., 55°C for 1 min., 72°C for 1 min. for 35 cycles, and finally incubated at 75°C for 10 min.

Aliquots were electrophoresed on agarose gels, stained with ethidium bromide, and the bands were photographed under ultraviolet light.

Results

We were interested in the mouse endothelioma cell line as a model for angiogenesis, but it had been poorly characterized for cytokine production. Therefore, PCR was used to prepare human DNA fragments from different cytokines, which then were spotted on a nylon membrane as shown in Fig. 1a (left panel). When probed with radioactive EOMA cDNA, the blot showed that, of the seven interleukin genes surveyed, only IL-3 was detected in substantial amounts (Fig. 1a, right panel). The presence of this mRNA was confirmed by Northern blot (Fig. 1b) and PCR (Fig. 1c). Because the latter is a sensitive technique, an IL-3 band also was found in unstimulated cells. However, a Western blot revealed a greater amount of IL-3 protein after stimulation with CAS (data not shown).

Discussion

This endothelioma cell line exemplifies a daunting problem, namely, since there are many cytokines, where does one start to look? We report here success in the use of a single extract of mRNA to screen for expression of multiple cytokine genes. We detected the presence of IL-3, which was unexpected, because human endothelial cells, stimulated with phytohemagglutinin and phorbol esters, do not express this cytokine (2). One of us (T.B.N.) had found previously that endothelial cells can support the growth of hematopoietic stem cells cultured with granulocyte-macrophage colony stimulating factor (3). Small amounts of IL-3 could be synergistic.

During our studies, a similar method for analysis of gene expression patterns was published (4). These workers spotted plasmid DNA on slot blots and detected c-myc, PCNA, and GAPDH

expressed by cardiac fibroblasts. Although their method may be slightly more sensitive because of larger plasmid targets (see below), our variation may be easier, since PCR fragments can be obtained directly from genes without cloning or multiple plasmid preparations.

In related experiments (by Y.Y.L. and F.M.R.), another cytokine also was detected, but did not show up on the multiple cytokine blots. Several explanations are possible. The PCR fragments that were used to make the blots were from human genes, and there could have been less nucleotide homology between other mouse cytokine sequences and human ones. A second possibility is that this screening procedure picks up only highly expressed messages, because the presence of the mouse housekeeping gene was not detected either. Finally, other mRNAs may not be produced in quantity at this time point of stimulation.

This method also could be used to follow the time course of expression for various cytokines, but some caveats should be kept in mind. Several factors limit the quantifiability of the detection system. The first is the unequal size of the inserts or PCR fragments between cytokines, which may vary by a few hundred nucleotides and lead to different amounts of DNA bound to each spot. The second is the variation in cDNA probe sizes, due to inherent disparities in mRNA length as well as variable efficiencies of cDNA synthesis. The longer or isotopically rich probes would give a higher signal. Thus interpretations should be made with care. Despite these uncertainties, it should be possible to increase the sensitivity of the assay. The challenge will be to amplify the signal of the cDNA probes without alteration of the relative ratios of the cytokine mRNAs.

In summary, we have developed a rapid screening system for cytokine gene expression. Our results indicate that the availability of multiple cytokine blots should be extremely useful to the scientific community.

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Legends

Fig. 1a: (Left panel) Grid for multiple cytokine blot. (Right panel) Autoradiograph after hybridization with ^{32}P-labeled cDNA from EOMA cells stimulated with concanavalin A supernatant (CAS).

Fig. 1b: Northern blots of EOMA RNA before (left lane) and after (right lane) stimulation with CAS. The probes were IL-3 (above), and G-3-PDH (below).

Fig. 1c: PCR with IL-3 primers and EOMA RNA. (L to R) Lane 1: unstimulated cells, Lane 2: stimulated with CAS, Lane 3: control IL-3 cDNA, Lane 4: control G-3-PDH.

a

GPDH	IL-1a	IL-1b
IL-3	IL-4	IL-5
IL-6	IL-11	

b

- IL-3

- G-3-PDH

c

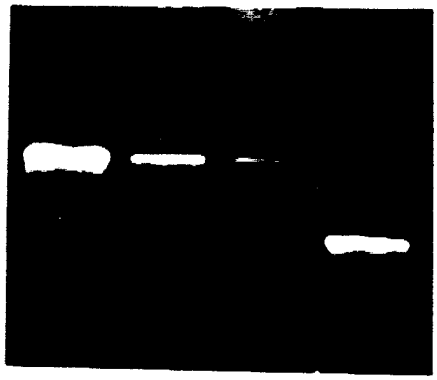

Fig 1a-c